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Development and Validation of an Intact Cell Assay for Protein Tyrosine Phosphatases Using Recombinant Baculoviruses

Wanda A. Cromlish, Paul Payette and Brian P. Kennedy*

Department of Biochemistry and Molecular Biology, Merck Frosst Center for Therapeutic Research, Merck Frosst Canada Inc., Pointe-Claire-Dorval, Quebec H9R 4P8, Canada

ABSTRACT. We have developed an intact cell assay to be used in the direct quantitation of protein tyrosine phosphatase (PTP) activity. Utilizing the baculovirus expression system, the assay readily allows for a direct activity readout for PTPs such as PTP1B or CD45. Infected *Sf*9 cells expressing either full-length PTP1B, full-length CD45, CD45 catalytic domain, or hCOX-1 (mock-infected) are harvested 29 hr post-infection, at which time cells are viable and the expressed proteins are processed, as well as localized to their predicted subcellular compartments. Assays are carried out in a 96-well format, with cells expressing the PTP of interest. Cells are preincubated with or without inhibitor and challenged with substrate, and the phosphatase activity is determined spectrophotometrically by monitoring the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol at OD_{405}. Documented PTP inhibitors have been used to validate this assay system. This study demonstrates that a direct readout of PTP activity in intact cells can be achieved, thus providing a useful cell-based screen for determining selective inhibitors of PTPs. BIOCHEM PHARMACOL **58**;10:1539–1546, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. baculovirus; protein tyrosine phosphatase; *p*-nitrophenyl phosphate; vanadate; pervanadate; inhibitor

PTPs† (EC 3.1.3.48) dephosphorylate tyrosyl residues, acting in concert with protein tyrosine kinases to control tyrosine phosphorylation of proteins in the cell. This reversible phosphorylation at tyrosyl residues has been found in many signal transduction pathways, such as those involved in the action of growth factors and in the control of cellular proliferation, differentiation, and metabolism [1, 2]. Although PTPs are very different in size and structural organization, they all contain a unique, highly conserved active site sequence, (I/V)HCXAGXXR(S/T)G [1–3]. Two prototypical PTPs of interest are CD45 and PTP1B. CD45, a transmembrane receptor-type PTP that activates protein tyrosine kinase p56lck and p59fyn, is involved in T cell [4, 5] and B cell [6] signaling. PTP1B is a nonreceptor PTP containing a single catalytic domain, and it has been implicated in insulin-resistance states and type 2 diabetes [3, 7–10].

Specific and selective PTP inhibitors would be of value as biological tools to reveal the function of individual phosphatases in cellular signal transduction pathways, and as disease-modulating drugs. To date, sodium orthovanadate (vanadate), pervanadate, and phenylarsine oxide are known to be PTP inhibitors; however, none of these exhibits a tyrosine phosphatase selectivity. Small peptides containing the hydrolytically stable difluorophosphonomethyl phenylalanine have been found to be inhibitors of PTP1B [11]. Available cell-based systems that can be used for identification of inhibitors have utilized downstream readouts for quantitation of PTP activity. A limitation of this type of assay is that inhibitors can interact at sites in the signal transduction pathway other than at the enzyme of interest, thus giving rise to a false positive result for the inhibitor. We previously developed and described an *in vitro* intact cell assay that utilized the baculovirus expression of COX in *Sf*9 cells for screening nonsteroidal anti-inflammatory drugs [12]. To discover cell-permeable, selective PTP inhibitors, we describe here an intact cell assay that has been developed utilizing the baculovirus expression system, where a PTP of interest is expressed in *Sf*9 cells and a direct readout of the phosphatase activity within these cells is obtained from the amount of hydrolysis of the cell-permeable substrate pNPP.

* Corresponding author: Wanda A. Cromlish, Department of Biochemistry and Molecular Biology, Merck Frosst Center for Therapeutic Research, Merck Frosst Canada Inc., P.O. Box 1005, Pointe-Claire-Dorval, Quebec H9R 4P8, Canada. Tel. (514) 428-3027; FAX (514) 428-8615; E-mail: wanda_cromlish@merck.com

† *Abbreviations*: PTP, protein tyrosine phosphatase; PTP1B-FL, full-length protein tyrosine phosphatase 1B; CD45-FL, full-length CD45; CD45-cat, CD45 catalytic domain; hCOX-1, human cyclooxygenase-1; pNPP, *para*-nitrophenyl phosphate; *Sf*9, *Spodoptera frugiperda*; MOI, multiplicity of infection; hpi, hours post-infection; and COX, cyclooxygenase.

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MATERIALS AND METHODS

Cell Culture

*Sf*9 (Invitrogen) cells were cultured in spinner flasks at 28° in Grace's supplemented medium (Gibco-BRL) with 10% heat-inactivated fetal bovine serum (Gibco-BRL) following the protocol of Summers and Smith [13].

Construction of Recombinant Baculovirus Transfer Vectors

The cDNA for PTP1B came from Dr. R. L. Erikson, Harvard University. The CD45 cDNA was obtained from Dr. Frank Jirik, University of British Columbia. The recombinant baculoviruses were prepared using the Bac-to-Bac Baculovirus Expression System (Gibco-BRL). Briefly, the genes of interest were cloned into the pFASTBAC donor plasmid, which had been engineered to include a FLAG sequence at the 5′ end of the cDNA. The FLAG sequence allows for easy identification and purification of the expressed protein of interest using the anti-FLAG M2 antibody (Intersciences Inc.). The resultant plasmids were transformed into competent DH10BAC *Escherichia coli* cells. Following transposition and antibiotic selection, the recombinant bacmid DNA was isolated from selected *E. coli* colonies and then used to transfect *Sf*9 insect cells. The virus found in the supernatant medium was amplified three times up to a total viral stock volume of 500 mL.

Production of Recombinant Proteins

Baculovirus infection of 500-mL spinner cultures of *Sf*9 cells was done essentially as described by Summers and Smith [13]. *Sf*9 cells at a density of $1–3 \times 10^6$ cells/mL were pelleted by centrifugation at 300 *g* for 5 min, the supernatant was removed, and the cells were resuspended at a density of 1×10^7 cells/mL in the appropriate recombinant viral stock (MOI of 10). Following gentle shaking for 1.5 hr at room temperature, fresh medium was added to adjust the cell density to $\sim 1 \times 10^6$ cells/mL, and the cells were cultured in suspension at 28° for the indicated times post-infection.

Cellular Fractionation and Whole Cell Extracts from Infected Sf9 Cells

At various times post-infection, aliquots were removed for analysis of protein expression by SDS–PAGE and western blot analysis. Cellular fractionation was performed as described [12]. Whole cell extracts were prepared from 1-mL aliquots of infected *Sf*9 cells at the specified times post-infection. Pelleted cells (300 *g*, 5 min) were washed once in PBS (4°), resuspended in 50 μL of water, and disrupted by freeze–thaw. Protein concentrations were determined by the Bradford assay (Pierce) with bovine serum albumin as the standard.

SDS–PAGE and Western Blot Analysis

Whole cell lysates and 100,000 *g* fractions (pellet or supernatant) were electrophoresed on Tris–glycine acrylamide denaturing gels (Novex), and the proteins were transferred electrophoretically to nitrocellulose [12]. The blots were incubated with a 1/2000 final dilution of FLAG M2 antibody (Intersciences Inc.) or a 1/500 dilution of anti-CD45 antibody (Transduction Laboratories). Immunoreactive proteins were visualized by using a 1/2000 dilution of anti-mouse Ig, horseradish peroxidase (Amersham) with development by enhanced chemiluminescence as described by the manufacturer (Amersham Life Science).

Intact Cell Assays and Inhibitor Titrations

*Sf*9 cells were infected with either PTP1B-FL, CD45-FL, CD45-cat, or hCOX-1 (mock-infected) [12] recombinant baculovirus. Infected cells were collected 29 hpi by centrifugation in a Beckman GS-6R at 460 rpm (48 *g*) for 5 min, washed once in assay buffer [Hanks' solution buffered with 15 mM HEPES, pH 7.4 (Sigma)] and recentrifuged at 300 rpm (21 *g*) for 10 min. The cells were resuspended gently in assay buffer and examined using a hemocytometer and microscope for cell density as well as viability by trypan blue exclusion. Assays were performed as follows using a Tomtec Quadra 96 pipetting robot, programmed to mix gently after each addition. Two hundred microliters of Hanks' solution containing 2×10^5 PTP-expressing cells was dispensed into each well of 96-well polypropylene plates. Inhibitor or DMSO vehicle (3 μL) was added to the appropriate well containing the cells. Following a 15-min inhibitor/DMSO preincubation at 37°, the cells were challenged with the indicated concentration of tissue culture-grade pNPP (Sigma-Aldrich Canada Ltd.) for 15 min. The cells were pelleted by centrifugation for 3 min at 410 *g* and 4°. Samples (100 μL) of the supernatants were transferred to fresh clear polystyrene 96-well plates, and the amount of hydrolysis of pNPP was determined spectrophotometrically at OD_{405}. The pNPP hydrolysis window obtained between PTP-expressing cells and mock-infected cells represents the amount of phosphatase activity due to the PTP of interest. Inhibitions were calculated by comparing pNPP hydrolysis of the inhibitor-treated PTP-expressing cells with that of DMSO-treated PTP-expressing cells.

The inhibitors studied included ouabain (Sigma-Aldrich), okadaic acid (Gibco-BRL), 30% H_2O_2 (Aldrich), phenylarsine oxide (Sigma), vanadate, and pervanadate. Vanadate (+5 oxidation state) and pervanadate [monoperoxo (VL) and diperoxo (VL_2) forms] were prepared as described [14].

RESULTS

Expression of PTP1B-FL, CD45-cat and CD45-FL

To set up an intact cell assay for a selective PTP inhibitor screen, initial characterization of the expression of phos-

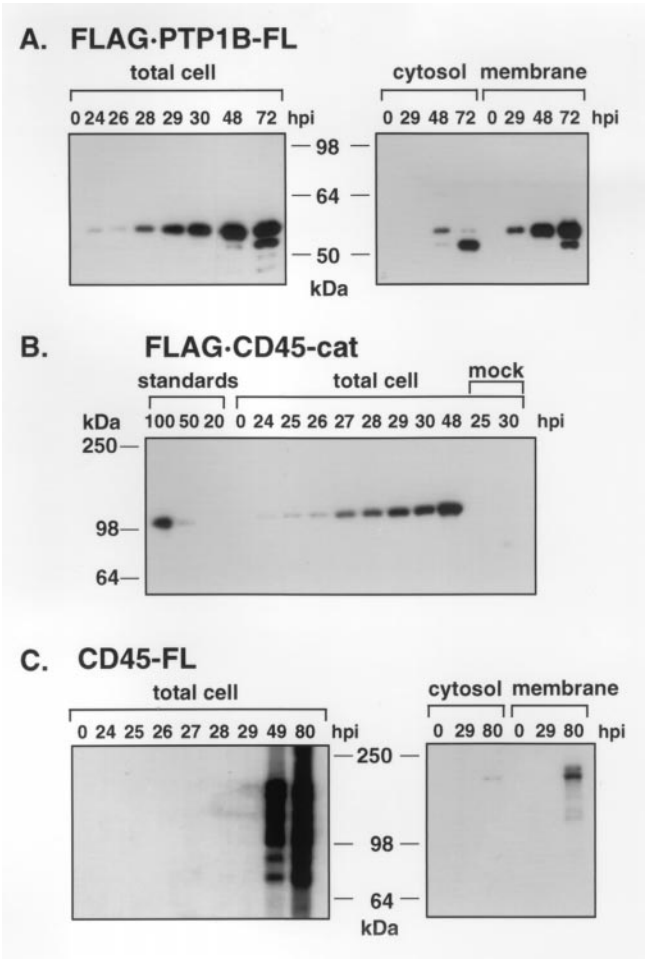

FIG. 1. Western blot analysis of PTP expression in *Sf*9 cells as a function of time in culture and subcellular localization. *Sf*9 cells were infected with recombinant virus for either FLAG-PTP1B-FL (A), FLAG-CD45-cat (B), or CD45-FL (C), and at the designated times post-infection (hpi), cells were harvested, and total cell lysates or 100,000 *g* cytosolic and membrane fractions were prepared. Proteins (6.5 μg/lane) from these preparations were separated by SDS–PAGE, transferred to nitrocellulose, immunoblotted with FLAG M2 antibody (A, B) or anti-CD45 antibody (C), and detected by chemiluminescence. Standards in B were 20, 50, and 100 ng of FLAG-CD45-cat purified from bacteria.

phatases from recombinant baculovirus-infected *Sf*9 cells was done. Western blot analysis (Fig. 1) was carried out on extracts from *Sf*9 cells infected with PTP1B-FL, CD45-cat, CD45-FL, or mock-infected (hCOX-1) cells to determine the extent of specific protein production, the time course of expression, and the subcellular localization of the individual phosphatases. Figure 1A shows that FLAG-tagged PTP1B-FL (~52 kDa) was expressed by 24 hpi, exponentially increased between 26 and 48 hpi, and then by 72 hpi began to be cleaved or degraded. At 29 hpi, PTP1B-FL was present only in the membrane fraction of the *Sf*9 cell. By 48 hpi, it also appeared intact in the cytosol. At 72 hpi, a 50-kDa cleavage product predominated in the cytosol, while the higher molecular mass form remained present in the membrane fraction. The expression pattern of FLAG-tagged CD45-cat was very similar to that of PTP1B and is shown in Fig. 1B. Exponential expression of the protein with an apparent molecular mass of 98 kDa was observed beginning at 24 hpi and continuing up to 48 hpi. Also included on this blot is the extract from mock-infected (hCOX-1) *Sf*9 cells at 25 and 30 hpi, where, as expected, no FLAG M2 antibody cross-reacted with any proteins. FLAG-tagged CD45-cat was localized cytosolically in the *Sf*9 cells, since FLAG affinity purification on an M2 antibody column yielded CD45-cat protein from the supernatant fraction of lysed cells (data not shown). The expression of full-length CD45, as shown in Fig. 1C, was significantly lower than that of the other two phosphatases, presumably reflecting the membrane localization of this protein. CD45-FL was just visible by western analysis of whole cell extracts at 29 hpi. At later stages of infection, 49 and 80 hpi, the ~150 kDa protein species predominated, but many presumable proteolytic degradation products cross-reacted with the anti-CD45 transmembrane antibody, especially when the gels were overloaded, as was the case shown here. Although no CD45-FL was visible at 29 hpi when the infected cells were fractionated, 80 hpi fractionation of the cells clearly showed that the full-length protein was localized mainly in the microsomal pellet, as expected, intact and undegraded.

All of the PTPs were expressed at various but detectable levels at 29 hpi. The cells at this time point are still highly viable [12], and therefore development of cell-based assays was pursued for these PTPs.

Intact Cell Assay Development

Initial characterization of the PTP insect cell-based assays was carried out at 29 hpi using PTP1B-FL and CD45-cat recombinant baculoviruses. Although many substrates were analyzed (data not shown), pNPP was chosen for its consistency and reproducibility of results. The rate of hydrolysis of pNPP, as measured spectrophotometrically at OD_{405}, correlated with substrate concentration, time of substrate challenge, cell number, viral titer, and tyrosine phosphatase protein expression. Using 2×10^5 PTP-expressing cells/well and varying the concentration of pNPP between 0 and 50 mM, the hydrolysis of the substrate by PTP1B-FL and CD45-cat during a 15-min incubation followed similar kinetics. Figure 2 illustrates the typical pNPP hydrolysis patterns by both phosphatases. In the absence of cells, increasing concentrations of pNPP led to a slight increase in the baseline absorbance at OD_{405}. Mock-infected (hCOX-1) cells contained background levels of PTPs and, therefore, exhibited increased hydrolysis of substrate with increasing substrate concentration. *Sf*9 cells infected with either PTP1B-FL or CD45-cat recombinant virus showed marked hydrolysis of pNPP at low substrate concentration, with maximal levels of substrate utilization occurring at 10 mM pNPP for PTP1B-FL and CD45-cat. At concentrations of pNPP greater than 10 mM for CD45-cat or 25 mM for PTP1B-FL, there appeared to be an effect of

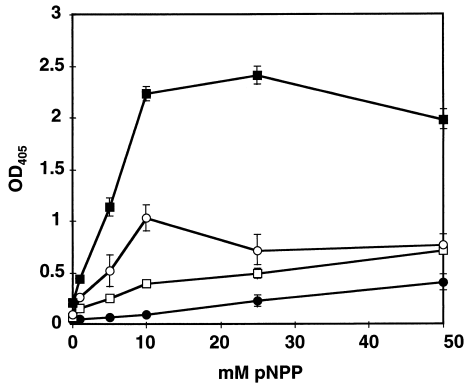

FIG. 2. Hydrolysis of pNPP substrate by intact *Sf*9 cells infected with recombinant baculovirus for FLAG-PTP1B-FL, FLAG-CD45-cat, or hCOX-1 (mock-infected). At 29 hpi, infected *Sf*9 cells (2 × 10^5 cells/well) expressing FLAG-PTP1B-FL (■), FLAG-CD45-cat (○), or mock-infected (□) were challenged with 0, 1, 5, 10, 25, or 50 mM pNPP for 15 min. Phosphatase activity was determined directly by spectrophotometrically measuring the amount of *p*-nitrophenol formed. The absorbance of various concentrations of pNPP in the absence of cells is also shown (●). Values represent means ± SEM for 4–10 experimental replicates.

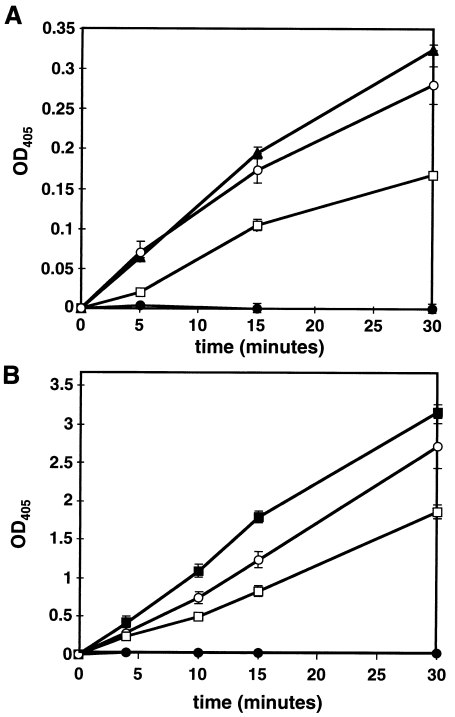

FIG. 3. Time course of hydrolysis of the substrate pNPP by CD45-cat and PTP1B-FL expressed in intact *Sf*9 cells. At 29 hpi, *Sf*9 cells (2 × 10^5 cells/well) expressing (A) FLAG-CD45-cat or (B) FLAG-PTP1B-FL were challenged with 0 (●), 5 (□), 10 (■), 25 (▲), or 50 (○) mM pNPP for 15 min. The extent of substrate hydrolysis by the phosphatase was determined spectrophotometrically at OD_{405}. Mock-infected *Sf*9 cell phosphatase activity has been subtracted. The data presented are a representative experiment showing the means ± range for duplicates.

substrate inhibition. The fact that the inhibition occurred at a higher concentration of pNPP for PTP1B-FL was probably due to the fact that more protein was expressed in these cells than in the CD45-cat expressing cells. The window between PTP hydrolysis and that of mock-infected cell hydrolysis represented the amount of substrate utilization due to the tyrosine phosphatase of interest.

The time course of substrate hydrolysis by *Sf*9 cells expressing CD45-cat or PTP1B-FL, in the presence of the indicated concentrations of pNPP, is shown in Fig. 3. pNPP hydrolysis was linear with respect to time up to 15 min of incubation, irrespective of the concentration of substrate used or phosphatase present in the *Sf*9 cells. At 30 min post-substrate challenge, the rate of hydrolysis began to fall off. The effect was more dramatic with the CD45-cat-expressing cells than with the PTP1B-FL-expressing cells, and presumably was related to the level of protein expressed and, therefore, the amount of substrate inhibition that occurred. For these reasons, a 15-min substrate hydrolysis period was chosen for further experiments. Figure 4 demonstrates that the rate of substrate hydrolysis was linear with respect to cell number in the assay. The cell number for further assays was set at 2 × 10^5 cells/well. The total output of PTP activity by the various expressed PTPs is shown in Fig. 5. Intact uninfected *Sf*9 cells, upon challenge with 10 mM pNPP, had a basal level of phosphatase activity as shown by the increase in the absorbance at 405 nm as compared with 10 mM pNPP/Hanks' alone. Intact mock-infected (hCOX-1) cells showed no difference with respect to phosphatase activity as compared with *Sf*9 cells that had not been infected. Intact *Sf*9 cells infected with either PTP1B-FL, CD45-cat, or CD45-FL recombinant baculoviruses had significantly increased pNPP hydrolysis rates of 7.6-, 5.7-, and 1.8-fold, respectively, above the mock-infected cell background. These levels of phosphatase activity correlated to the relative amount of protein de-

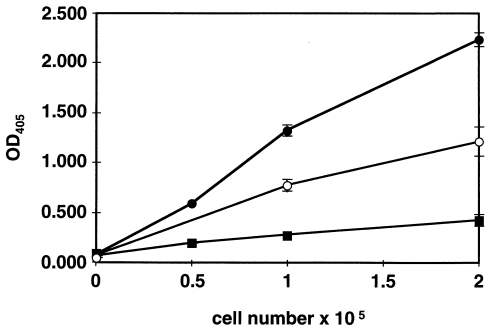

FIG. 4. Substrate hydrolysis profiles of intact *Sf*9 cells infected with recombinant CD45-cat, PTP1B-FL, or hCOX-1 (mock) baculovirus as a function of assay cell number. FLAG-PTP1B-FL (●), FLAG-CD45-cat (○), or hCOX-1 (■) infected *Sf*9 cells at 0.5, 1, or 2 × 10^5 cells/well were challenged with 10 mM pNPP for 15 min, and the amount of hydrolyzed substrate was measured at OD_{405}. The data presented for 1 or 2 × 10^5 cells/well represent the means ± SEM for 4–7 replicates per point, whereas the values for 0 or 0.5 × 10^5 cells/well are the means ± range of duplicates.

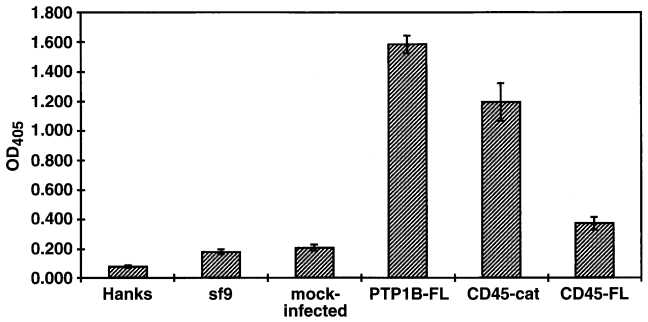

FIG. 5. Typical substrate hydrolysis profiles of intact *Sf*9 cells infected with recombinant baculoviruses for PTP1B-FL, CD45-cat, CD45-FL, or hCOX-1 (mock). Infected cells harvested at 29 hpi were washed and resuspended in Hanks'/HEPES; 2×10^5 cells/well were challenged with 10 mM pNPP for 15 min, and the amount of *p*-nitrophenol was detected at OD_{405}. The data presented represent means ± SEM for 6–11 experiments except for the value for the *Sf*9 cells, which is the mean ± range of duplicates.

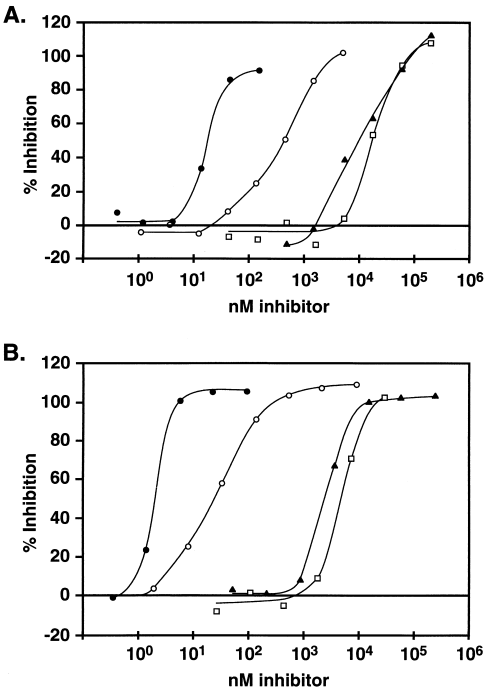

FIG. 6. Representative inhibition profiles for known inhibitors of PTP activity for PTP1B-FL (A) or CD45-cat (B) expressed in *Sf*9 cells at 29 hpi. The infected cells were washed and resuspended in Hanks'/HEPES buffer, pH 7.4, at 37°, where 2×10^5 cells/well were preincubated with various concentrations of vanadate (○), phenylarsine oxide (□), H_2O_2 (▲), or pervanadate (●) for 15 min, and then challenged for 15 min with 10 mM pNPP. The amount of *p*-nitrophenol was determined at OD_{405}. Inhibitions were calculated by comparing pNPP hydrolysis of the inhibitor-treated PTP-expressing cells with that of DMSO-treated PTP-expressing cells.

tected at 29 hpi in the western blot analysis (Fig. 1), with PTP1B-FL being most highly expressed, followed by the expression level of CD45-cat and then the much lower level of expression of CD45-FL. In the case of PTP1B-FL and CD45-cat, the amount of hydrolysis of pNPP by the enzymes inside the intact *Sf*9 cells represented ~30% of the total hydrolysis that could occur if the cells were disrupted and the enzymes were released to be in direct contact with the substrate (data not shown).

The assay conditions used for further experiments were as follows. The infected *Sf*9 cells expressing either PTP1B-FL, CD45-cat, or CD45-FL were harvested at 29 hpi by gentle centrifugation and resuspended in HEPES-buffered Hanks' solution, pH 7.4, at 37°. The cells (2×10^5 PTP-infected) were challenged with a final concentration of 10 mM pNPP for 15 min and spun out at 4°, and the amount of substrate hydrolysis was determined by a spectrophotometer at OD_{405}.

Inhibitor Characterization

The potency and selectivity of various compounds to inhibit the PTP activity arising from the expressed phosphatases in the intact infected *Sf*9 cells was addressed. The inhibitors were preincubated with the infected *Sf*9 cells for 15 min prior to the 15-min challenge with 10 mM pNPP. The inhibitors tested included ouabain, an inhibitor of Na-K-ATPase known also to exhibit *p*-nitrophenyl phosphatase activity; okadaic acid, a serine/threonine phosphatase inhibitor; and H_2O_2, phenylarsine oxide, vanadate, and pervanadate, which represent four documented PTP inhibitors. Figure 6 shows representative IC_{50} profiles of some of the inhibitors on PTP activity of CD45-cat and PTP1B-FL. The complete data are summarized in Table 1. Neither ouabain at 1 mM nor okadaic acid at 6.7 μM had any effect on CD45-cat or the background phosphatases found in infected *Sf*9 cells. As predicted, H_2O_2 and phenylarsine oxide showed limited potency and selectivity in this intact cell assay, with IC_{50} values in the range of 7–16 μM. Vanadate was very effective at inhibiting the expressed PTPs, with slightly more selectivity (4.5x) towards CD45 than PTP1B-FL. It was also not as potent on the background tyrosine phosphatases in mock-infected *Sf*9 cells, with an IC_{50} of 1.6 μM. Pervanadate was the most potent inhibitor, with IC_{50} values between 5 and 20 nM against CD45 and PTP1B-FL. Pervanadate is a general term for the variety of complexes formed between vanadate and H_2O_2. At the concentrations of vanadate and H_2O_2 used in these experiments, the major species added to the cells were the monoperoxo (VL) and diperoxo (VL_2) forms. Pervanadate inhibits PTPs by irreversible inactivation of the active site cysteine, a mechanism distinct from the competitive inhibitory nature of vanadate [14].

DISCUSSION

The role of PTPs in the modulation of signal transduction by tyrosine kinase-containing receptors and oncogene products has been gaining more investigative attention. PTP1B, a cytosolic nonreceptor PTP, was one of the first PTPs identified. Recently, targeted mutagenesis of PTP1B

TABLE 1. IC_{50} Values of known inhibitors of PTP activity for PTB1B-FL, CD45-cat, CD45-FL, or hCOX-1 (mock) expressed in *sf*9 cells at 29 h post-infection

	IC_{50} (μM)			
Compound	**PTP1B-FL**	**CD45-cat**	**CD45-FL**	**Mock-infected**
Ouabain	ND	> 1000	ND	> 1000
Okadaic acid	ND	> 6.7	ND	> 6.7
H_2O_2	10.9 ± 0.6	9.9 ± 1.9	15.4	ND
Phenylarsine oxide	16.3 ± 4.4	10.4 ± 0.3	7.2	ND
Vanadate	0.29 ± 0.05	0.07 ± 0.01	0.06 ± 0.015	1.6
Pervanadate	0.02 ± 0.005	0.01 ± 0.004	0.004 ± 0.0007	ND

The infected cells were washed, resuspended in Hanks'/HEPES buffer, pH 7.4, at 37°, where 2×10^5 cells/well were preincubated with various concentrations of the indicated inhibitors for 15 min, and then challenged for 15 min with 10 mM pNPP. The amount of hydrolysis product, *p*-nitrophenol, was determined spectrophotometrically at OD_{405}. The data represent means ± SEM for 3–9 separate titration experiments. ND = not determined.

has revealed that it plays a role in both insulin signaling and fat metabolism, making it a potential therapeutic target for the treatment of type 2 diabetes and obesity [7]. CD45 is a transmembrane receptor-type PTP expressed on all hematopoietic cells except erythrocytes [15] and is required for normal T and B cell signaling [4–6]. CD45 has the potential for therapeutic benefits in the area of immunosuppression.

The successful development of intact *Sf*9 cell-based assays for COX-1 and COX-2 [12] that could identify potent and isozyme-selective nonsteroidal anti-inflammatory drugs prompted us to again investigate the use of the baculovirus system to define potent, selective, and cell-permeable inhibitors for specific intracellularly expressed phosphatases. An alternative method to discriminate between the activity of a PTP of interest and other phosphatase activity within the cell is to monitor some downstream readout parameter. For example, in the case of CD45, the amount of interleukin-2 secreted by Jurkat cells can be used as a measure of the activity of this PTP. We describe here a "mix and read" intact cell assay for PTP activity, which eliminates the potential of inhibitors to interfere at sites in the signal transduction pathway other than at the enzyme of interest. The PTP of interest is overexpressed in *Sf*9 cells for a short period of time and challenged with inhibitor, then substrate, followed by direct readout of inhibition.

The *Sf*9 insect cell line used in the baculovirus expression system is derived from the ovarian tissue of the fall armyworm *Spodoptera* [13]. Infection of this cell line with recombinant baculoviruses for PTP1B and CD45 results in the expression of these proteins. Note also that the genome of the baculovirus *Autographa californica* encodes a 19-kDa PTP [16]. *Sf*9 cells appear to offer the cellular environment and factors necessary for both PTP1B and CD45 phosphatase activity. Although insoluble recombinant protein (denatured aggregates) can be a phenomenon in the baculovirus expression system, our experience with these phosphatases and cyclooxygenases [12] has shown that all of the membrane-expressed proteins are localized properly in the *Sf*9 cells as the active proteins of interest. The level of activity has correlated consistently with the amount of protein produced. The subcellular localization of the expressed PTPs was as expected. The catalytic domain of CD45 (CD45-cat) was expressed at high concentrations in the cytosolic fractions of the cell extracts prepared, since this protein contained no transmembrane domain as found in the full-length CD45. CD45-FL was expressed minimally (as compared with the general expression level of many proteins) in the membrane fraction of *Sf*9 cells. This was probably due to the fact that it is a transmembrane protein and, therefore, proceeds through the Golgi prior to insertion in the plasma membrane. It is interesting to note that the level of expression of the full-length form of CD45 at 29 hpi was the same as that found in an equivalent number of Jurkat cells, which express very high amounts of CD45 per cell surface area (data not shown). Full-length PTP1B is reported to be anchored in the endoplasmic reticulum at its C-terminal [17]. PTP1B-FL was expressed at very high levels and was found to be associated with the membrane fraction at early time points post-infection. By 48 hpi, a time when the viability of the infected cells begins to decline, PTP1B-FL also was found in the cytosol, and at greater than 72 hpi, a 50-kDa N-terminal fragment appeared both in the membrane and cytosolic fractions, presumably due to proteolytic breakdown of the protein.

For the PTP *Sf*9 cell-based assay to be useful in screening for potential new therapeutic agents, it was necessary to harvest the cells at a time point when sufficient amounts of the protein of interest were produced, yet the cells were healthy and viable prior to the deleterious effects associated with the later stages of the viral infection. The infected *Sf*9 cells were harvested at 29 hpi, a point when each of the PTPs was fully post-translationally modified, properly targeted, and active. Using intact *Sf*9 cells expressing PTP1B or CD45, we have characterized known PTP inhibitors. High throughput screening was instituted by assaying in 96-well plates with mechanization facilitated by a Tomtec Quadra 96 pipetting device.

The inhibition of PTP activity by various compounds was detected by measuring the change in the hydrolysis of pNPP. This substrate has a K_m in the millimolar range, suggesting that inhibitors identified using pNPP may be competing for the binding site with lower affinities. The requirement of our assay, however, was that a cell-perme-

able, hydrolyzable substrate was needed. Although many substrates were tried, pNPP was chosen because it gave the most consistent results. Uptake of phosphate into eukaryotic cells is very important and vital for metabolic requirements of growth or replication. The substrate, pNPP, is presumed to be taken up into the *Sf*9 cells by a phosphate transporter. In general, it has been found that pNPP hydrolysis parallels PTP activity, although the pH optimum for the hydrolysis of pNPP by PTP1B is slightly more acidic than for its PTPase activity [18]. It should be pointed out that no one assay is relied upon for the development of a drug and that the cell-based assay described here would be part of a multitude of *in vitro* and *in vivo* assays that an inhibitor would encounter during the drug development process. Each assay would use a different substrate. The rate of hydrolysis of pNPP in the *Sf*9 cell assay correlated well with the viral titer used to infect the cells, as well as the cell number and substrate concentration in the assay. The window of phosphatase activity seen between the PTP of interest and the background activity in mock-infected cells correlated with the amount of protein expressed. PTP1B-FL and CD45-cat were the most highly expressed of the three PTPs, and hence revealed a 5- to 6-fold and 3- to 4-fold window of pNPP hydrolysis activity, respectively. CD45-FL was barely detected by western blot and concomitantly showed a 1.5-fold window of activity. For any cell-based assay to be useful in drug discovery, a reproducible window of activity must be achieved that is greater than 2-fold. PTP1B-FL and CD45-cat expressing cells easily fulfilled this criterion, and, as revealed by the IC_{50} data, gave rise to reliable and reproducible results. The very small window of phosphatase activity due to the low expression level of CD45-FL in the *Sf*9 cells did not allow them to be useful for screening purposes. Replicates exhibited a high degree of error, and IC_{50} values from experiment to experiment could shift by as much as 3-fold.

The profiles of the inhibitors tested were as expected. Sigmoidal IC_{50} curves for this assay system were obtained, with replicates within and between experiments being very reproducible. The tighter the reproducibility of an assay system, the better the structure–activity relationships that can be denoted. Na-K-ATPase is known to exhibit pNPP hydrolyzing activity. To demonstrate that the amount of *p*-nitrophenol detected in our assay was due to phosphatase activity, ouabain, an inhibitor of Na-K-ATPase, was tested and showed no inhibition up to 1 mM for both the background infected cells and CD45-expressing cells. Serine/threonine phosphatases were not hydrolyzing pNPP, since okadaic acid [19–21] had no effect. H_2O_2 has been shown to inactivate recombinant PTP1B *in vitro* by oxidizing its catalytic site cysteine [22]. Direct exposure of cells to H_2O_2 activates signal transduction pathways by increasing protein tyrosine phosphorylation [23–25]. The testing of H_2O_2 in our cell-based assay indeed showed that nonselective inhibition of PTP1B or CD45 in the IC_{50} range of 10–15 μM was obtained. Phenylarsine oxide is a documented membrane-permeable inhibitor of protein phosphotyrosine phosphatases [26]. Our results testify that it is a non-potent, non-selective inhibitor of PTP1B and CD45 expressed within *Sf*9 cells. Vanadate is a general PTP inhibitor [27] that has been found to be competitively and reversibly bound at the active site [14]. It has insulin-mimetic properties [28] and has been shown in human clinical trials to be potentially useful in treating non-insulin-dependent diabetes mellitus [29]. Vanadate was chosen as a gold standard to validate this assay. It is much more potent than H_2O_2 and phenylarsine oxide at inhibiting the expressed phosphatases. It appeared to be slightly more selective for CD45 than PTP1B. Pervanadate, a complex of vanadate and H_2O_2, is also an insulin-mimetic and has been documented as being more potent at increasing the levels of cellular tyrosine phosphorylation [30–34]. It was found to be an irreversible inhibitor of PTP1B [14]. Our data correlate with and substantiate these observations. Pervanadate was 7–15 times more potent than vanadate at inhibiting CD45 and PTP1B expressed in the *Sf*9 cell assay. However, there was no selectivity to this inhibition.

The baculovirus expression system can supply recombinant protein for enzymatic characterization, x-ray crystallographic studies, *in vitro* purified enzyme and microsomal assays, and, as reported previously [12] and here, reliable cell-based assays. The PTP1B and CD45 assays presented in this study illustrate that it will be possible to adapt this assay to any PTP and in so doing provide an opportunity to uncover potentially useful inhibitors as biological research tools in understanding signal transduction pathways and for the treatment of many, yet to be identified, therapeutic targets.

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