Functional Expression of the Human Growth Factor Activatable Na^+/H^+ Antiporter (NHE-1) in Baculovirus-Infected Cells†

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ABSTRACT: We constructed a recombinant baculovirus, based on *Autographa californica* nuclear polyhedrosis virus, containing the human Na^+/H^+ antiporter cDNA under control of the polyhedrin promoter. When infected with this recombinant baculovirus, the Sf9 cell line, derived from *Spodoptera frugiperda*, expresses a fully functional Na^+/H^+ antiporter as measured by the generation of an amiloride-sensitive Na^+ influx in response to an acid load. The Na^+/H^+-exchange activity, not detectable in Sf9 cells, emerges 18 h after infection and continues to increase over the next two days to reach a maximal value about 20-fold higher than in cultured mammalian fibroblasts. Parallel to this activity, infected cells express a single immunoreactive polypeptide of 85 kDa that represents a non-glycosylated form of the 110-kDa mature human antiporter. We estimated that only 10% of the expressed protein is in a functional state. Not only is the antiporter expressed in insect cells phosphorylated, but also, like in mammalian cells, phosphorylation is increased in response to phorbol esters and okadaic acid. Moreover, tumor promoters apparently modify the same antiporter site in both insect and mammalian cells. We conclude that, with this high level of functional expression and apparently conserved signaling machinery, the present system opens the way to the biochemistry of the transporter including identification of the growth factor stimulated phosphorylation sites.

The concentration of cytoplasmic H^+, like that of other ions, is finely regulated in eukaryotic and prokaryotic cells by various types of membrane transporters, channels, and pumps. The concentration of some ions must be adjusted within the limit of a narrow window to permit extracellular signals to propagate within the cell and essential metabolic and physiological processes to operate. Intracellular pH is regulated in vertebrate cells by at least three membrane components, which include an amiloride-sensitive Na^+/H^+ antiporter, Na^+-dependent and -independent Cl^-/HCO^-_3 antiporters, and presumably a slow active H^+-extruding system identified in some cells as a H^+-ATPase [for reviews see Aronson and Boron (1986), Grinstein (1988), and Haussinger (1988)]. In vertebrate cells, the antiporter operates as a major H^+-extruding system driven by the inwardly directed Na^+ chemical gradient and activated through an allosteric internal proton regulatory site dubbed the "H^+ sensor".

This membrane transporter has received a great deal of attention in the past few years because (i) one of the earliest actions of growth factors on quiescent cells is an activation of the Na^+/H^+ antiporter [for reviews see Pouysségur (1986), Moolenaar (1986), and Grinstein et al. (1989)] and (ii) a link between the regulation of pH_i and the control of cell growth has been firmly established (Pouysségur et al., 1984; 1985; Perona et al., 1990; Gillies et al., 1990). Growth factors are thought to induce a covalent modification of the antiporter, resulting in an apparent increased affinity of the intracellular "H^+ modifier site" (Moolenaar et al., 1983; Paris et al., 1984; Grinstein et al., 1985). This biochemical change is remarkably "universal" in that it occurs in response not only to growth factors but also to various activating agents such as sperm, neurotransmitters, chemotactic peptides, phorbol esters, mitogenic lectins, and hyperosmotic stress (Grinstein et al., 1989).

Recently, we reported the molecular cloning of this growth factor activatable Na^+/H^+ antiporter (Sardet et al., 1989). This gene referred to as NHE-1[1] (Na/H exchanger 1) is ubiquitously expressed and present in the basolateral membranes of polarized epithelial cells (Sardet et al., 1990b; Tse et al., 1991). It encodes a phosphoglycoprotein of 110 kDa containing 10 putative transmembrane segments followed by a hydrophilic cytoplasmic stretch of 315 residues. A functionally and structurally related second form, NHE-2, that is expressed at the apical surface of intestine and kidney epithelial cells has just been isolated (M. Tse, J. Pouysségur, and M. Donowitz, manuscript in preparation). Further, we demonstrated that mitogenic activation of resting fibroblasts with epidermal growth factor, thrombin, phorbol esters, or serum stimulated the phosphorylation of NHE-1 in a time course that parallels its activation (Sardet et al., 1990a, 1991). Interestingly, all activating agents apparently phosphorylate the same phosphopeptides at serine residues. Therefore, the NHE-1 isoform of Na^+/H^+ antiporter provides an attractive model to analyze post-receptor-induced chemical modification and to resolve the integration of distinct transmembrane signals into a common cellular response.

Although gene manipulation is a powerful approach for identifying functional domains of the transporter, it cannot resolve all the questions. An understanding of the structural organization and the definition of key phosphorylation sites and cytoskeletal interacting domains require purification of a functional transporter. NHE-1, like most plasma membrane channels and transporters, is expressed in minute amount in tissues and cultured cells. This prompted us to use the baculovirus–Sf9 expression system that has been successfully used

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[1] Abbreviations: NHE, Na/H exchanger; AcNPV, *Autographa californica* nuclear polyhedrosis virus; MPA, methylpropylamiloride; PMA, phorbol myristate acetate.

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for high expression levels of functional proteins (Luckow & Summers, 1988; Miller, 1988).

We describe here the cloning of the human NHE-1 cDNA isoform into *Autographa californica* nuclear polyhedrosis virus (AcNPV) and its functional expression in the insect cell line Sf9 derived from *Spodoptera frugiperda*. Functionality was judged by the emergence of a H^+-induced amiloride-sensitive Na^+ influx in infected cells, which had the features of the human growth factor activatable Na^+/H^+ exchanger. More importantly, the human antiport protein is phosphorylated in Sf9 cells, and tumor promoters stimulate its phosphorylation apparently on the same site as in mammalian fibroblasts. This finding strongly validates the use of this expression system to identify the phosphorylation sites and the specific activated kinases.

Experimental Procedures

Construction of Recombinant Baculovirus Transfer Vector. The transfer vector pEV-55-mod was kindly provided by Dr. Lois K. Miller (University of Georgia, Athens, GA). The human Na^+/H^+-exchanger cDNA (NHE-1 isoform) containing the entire open reading frame and 53 bp of the 5′ flanking sequence was excised from the cDNA clone c28 (Sardet et al., 1989) by digestion with *Xho*I and *Kpn*I, purified, and inserted at the *Xho*I/*Kpn*I sites of pEV-55-mod. Recombinant plasmid pEV–NHE-1 DNA was prepared as described earlier (Maniatis et al., 1982) and purified by CsCl density gradient centrifugation.

Cells and Viruses. The *S. frugiperda* (Sf9) cell line was grown at 27 °C in TC100 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum. Propagation of wild-type and recombinant baculoviruses was obtained following inoculation of Sf9 cells at a multiplicity of infection (moi) of 10 pfu/cell. After 1 h at room temperature, the virus inoculum was removed and replaced by complete growth medium. The Chinese hamster lung fibroblast cell line, pEAP, was cultivated as previously described (Pouyssegur et al., 1984). It is an antiporter high expressor that derives from the antiporter-deficient clone PS120 (Pouyssegur et al., 1984) following transfection of the human cDNA under the control of the SV40 early promoter.

Na^+/H^+ Antiporter Probe. A 1914-bp *Bam*HI restriction fragment of the human Na^+/H^+-exchanger cDNA (Sardet et al. 1989) was radiolabeled with [α-^{32}P]dCTP with use of random primer extension.

Transfection and Selection of Recombinant AcNPV. Wild-type AcNPV DNA was prepared from purified extracellular virus as described previously (Summers & Smith, 1987). Recombinant baculoviruses were generated by co-transfecting Sf9 cells with wild-type baculovirus AcNPV viral DNA (1 μg) together with 50 μg of the Na^+/H^+ antiporter recombinant transfert vector pEV–NHE-1 by the calcium phosphate coprecipitation method (Summer & Smith, 1987). Culture supernatants were harvested 4 days after transfection. Recombinant baculoviruses were identified by a limiting dilution procedure (Fung et al., 1988) and hybridization with the NHE-1-specific probe (*Bam*HI fragment of c28 cDNA). Recombinant viruses were further purified by two similar rounds of screening at a moi of 2 and 0.5 pfu/well. Final purification was obtained by standard plaque assay procedures (Summers & Smith, 1987) and picking of occlusion-negative plaques.

Western Blotting and SDS–PAGE Analysis of Proteins. Sf9 cells were infected with recombinant baculovirus at a moi of 10. At various times postinfection, cells were collected and lysed in Laemmli's sample buffer (Laemmli, 1970). Proteins were resolved by SDS–PAGE and transferred to nitrocellulose membrane (hybond C Amersham). The nitrocellulose filter was probed with polyclonal affinity-purified rabbit anti-Na^+/H^+-exchanger antibody (Sardet et al., 1990a) and then immunodetected with a peroxidase-labeled goat anti-rabbit IgG antibody.

Labeling of Infected Cells. Attached cells on 60-mm Petri dishes were infected at a moi of 10 for 5 h. Thereafter, cells were starved overnight by replacing the complete medium with TC100 without serum. After 18 h of infection, cells were washed once in bicarbonate- and phosphate-free DMEM medium containing 20 mM Hepes (pH 6.3) and then incubated for 6 h in the same medium containing 100 μCi/mL [^{32}P]orthophosphate. When [^{32}P]orthophosphate-labeled proteins were prepared for tryptic peptide mapping analysis, the specific radioactivity was raised to 0.5 mCi/mL. After required treatment with tumor promoters, the incubation medium was removed and the dishes were frozen in liquid nitrogen.

Na^+/H^+ Antiporter Immunoprecipitation and Phosphopeptide Mapping. The frozen cell monolayers were scraped and resuspended in 500 μL of ice-cold buffer containing 50 mM Hepes/NaOH (pH 7.4), 150 mM NaCl, 3 mM KCl, 25 mM sodium pyrophosphate, 10 mM adenosine triphosphate, 5 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM *o*-phenanthroline, and 1 mM iodoacetamide and centrifuged for 15 min at 100000*g*. The pellets were resuspended in the same buffer containing 1% Nikkol (Nikko Chemicals, Japan). Solubilization of protein and immunoprecipitation were carried out as previously described (Sardet et al., 1990).

For phosphopeptide mapping, [^{32}P]orthophosphate-labeled proteins were eluted from SDS–PAGE gel slices by overnight incubation of homogenized acrylamide gel at 37 °C in 50 mM NH_4CO_3 (pH 8)/1% SDS/1 mM DTT/1 mM PMSF. Proteins in the supernatant were coprecipitated with 100 μg of bovine serum albumin in the presence of 15% TCA. After centrifugation the pellet was washed 3 times with 50% ethanol/50% ether to remove any remaining SDS and TCA. The sample was resuspended in 500 μL of 50 mM ammonium bicarbonate (pH 8) and digested twice with 20 μg of (tosylamino)-2-phenylethyl chloromethyl ketone- (TPCK-) treated trypsin for 15 h with shaking at 37 °C. The resulting tryptic peptides were lyophilized and separated by thin-layer electrophoresis at pH 1.9 for 1 h at 850 V in the first dimension and by thin-layer chromatography in the second dimension as described previously (Cooper & Hunter, 1981). Phosphopeptides were visualized by autoradiography.

^{22}Na Uptake Studies. Cells were plated in 24-well dishes and infected as described above. At various times after infection, cells were incubated for 30 min at 28 °C in 10 mM Hepes (pH 7.5)/30 mM NH_4Cl/90 mM choline chloride (Franchi et al., 1986), quickly washed once (washing shorter than 30 s) with 120 mM choline chloride/15 mM Hepes (pH 7.4), and incubated at 28 °C in ^{22}Na uptake medium: 120 mM choline chloride, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes (pH 7.4), 1 mM ouabain, and 1 μCi/mL of carrier-free ^{22}NaCl. Influx of ^{22}Na was stopped by rinsing the cell monolayers four times with phosphate-buffered saline solution at 0 °C. Cells were solubilized in 0.1 N NaOH, and radioactivity was assayed by liquid scintillation counting. Protein concentrations were estimated by the procedure of Lowry et al. (1951) with crystalline bovine serum albumin as a standard.

Results

Construction of a Recombinant Baculovirus Containing a

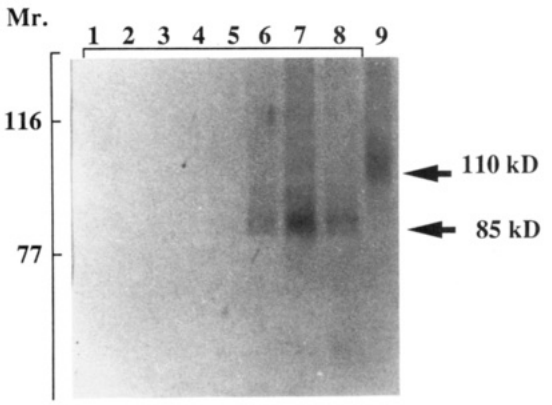

FIGURE 1: Immunological detection of the Na^+/H^+ antiporter in insect cells infected with recombinant baculovirus. Sf9 cells were infected as described under Experimental Procedures. At the required time, cells were washed twice with PBS and proteins were solubilized in Laemmli's buffer. Proteins from 10^5 cells were electrophoresed on SDS–PAGE and transferred to hybond C. Na^+/H^+ antiporter was detected by use of an anti-Na^+/H^+-antiporter antibody as previously described (Sardet et al., 1990). Lane 1: noninfected cells. Lane 2: Sf9 cells infected for 24 h with wild-type virus. Lanes 3, 4, 5, 6, 7, 8: cells infected for 5, 10, 15, 18, 24, and 48 h, respectively. Lane 9: pure membranes from hamster fibroblasts overexpressing 15-fold the human NHE-1 protein. Standard molecular mass markers are shown on the left.

Human NHE-1 Gene. To introduce the human NHE-1 gene into the genome of wild-type AcNPV, the recombinant transfer vector pEV–NHE-1 was constructed as outlined under Experimental Procedures. Plasmid pEV–NHE-1 contains an NHE-1 cDNA, including the full-length coding region, which is flanked by 53 bp of the 5′ untranslated region and the entire 3′ noncoding region of c28, under the control of the strong polyhedrin promoter. Plasmid pEV–NHE-1 and wild-type AcNPV DNA were cotransfected in the permissive host cell line Sf9 (Summers & Smith, 1987). Homologous recombination between these DNA sequences gave rise to recombinant BV–NHE-1 baculoviruses that were detected initially by hybridization with a NHE-1 cDNA probe and then by visual discrimination of recombinant viruses as occlusion-negative plaques.

Expression of Na^+/H^+ Antiporter in Sf9 Cells. The expression of recombinant Na^+/H^+ antiport protein following infection of Sf9 cells was monitored by immunoblotting with use of an antibody directed against the Na^+/H^+ antiporter C-terminal region (Sardet et al., 1990a). The experiment of Figure 1 describes a time-course experiment in which extracts of Sf9 cells infected with the recombinant BV–NHE-1 baculovirus were analyzed for the expression of the Na^+/H^+ antiporter. After 18 h of infection, the antibody detects expression of a single polypeptide of 85 kDa, the level of which appears maximal at 24 h. The amount of recombinant Na^+/H^+ antiport protein detected in Sf9 cultures decreased after 24 h of infection. This resulted from extensive cell death occurring at that time and presumably resulting from the toxic effect of the overexpressed antiporter. In hamster fibroblast, an overexpressed human antiporter migrates as a 105–110-kDa polypeptide in SDS–PAGE. This difference in mobility indicates that the antiporter expressed in Sf9 cells is not or is poorly glycosylated. Indeed, the 85-kDa expressed polypeptide comigrates with the human Na^+/H^+ antiporter cRNA translated in vitro by rabbit reticulocyte lysates or with the 110-kDa fibroblast polypeptide treated with endoglycosidase F and neuraminidase (data not shown; Sardet et al., 1990).

We next analyzed the structural and functional features of the Na^+/H^+ antiporter expressed in Sf9 cells. We therefore measured the proton-activated and amiloride-sensitive $^{22}Na^+$ influx as a function of infection time. Figure 2 shows that the amiloride-sensitive Na^+ influx is not detectable in noninfected cells but emerges 16–18 h following infection. The specific activity rapidly increases between 18 and 24 h to reach a maximal value thereafter. This Na^+ influx takes place only in response to intracellular acidification and is totally suppressed by amiloride, a fundamental characteristic of the Na^+/H^+ antiporter. We calculated, on the basis of the amiloride-sensitive $^{22}Na^+$ initial rates, that the maximal functional expression obtained in Sf9 cells (20 cpm/(μg·min)) represents 20–30 times that observed in normal mammalian fibroblasts such as the hamster cell line CCL39 (0.07 cpm/(μg·min)). Now, to evaluate whether the protein expressed in Sf9 cells is entirely functional, we compared the Na^+/H^+ antiport activities (V_{Max} values) and the amount of NHE-1 protein expressed in insect cells and in hamster fibroblasts transfected with the human gene (pEAP cell line). Figure 3a shows the antiport activity as a function of the concentration of NH_4^+ used for acid loading. In both cell types the V_{Max} value is obtained above 30 mM NH_4^+ and is 5 times higher in Sf9 cells than in the transfected hamster fibroblasts pEAP. However, comparison of the proteins by immunoblotting reveals that infected Sf9 cells express 150–200-fold more protein than pEAP cells (Figure 3b). The dose–response curve for the inhibition of Na^+/H^+ exchange by the potent amiloride analogue, MPA, is presented in Figure 4. The $K_{0.5}$ for MPA is around 10 nM, a value similar to that found for the human Na^+/H^+ antiporter expressed in hamster fibroblasts (unpublished results). These results indicate that the 85-kDa polypeptide, expressed in insect cells, is correctly inserted in the membrane and, in spite of a glycosylation defect, it functions invariably as in its natural environment. However, only a small fraction of the antiport protein expressed (10%) participates in the transport in insect cells.

FIGURE 2: ^{22}Na uptake in insect cells as a function of infection time by recombinant baculovirus. ^{22}Na uptake (3-min uptake values) were conducted in H^+-loaded cells as described under Experimental Procedures. The open squares represent ^{22}Na flux in the presence of 100 μM methylpropylamiloride (MPA). This representation is the mean from two independent experiments, each performed in triplicate.

Phosphorylation of the Na^+/H^+ Antiporter. The Na^+/H^+ antiporter is phosphorylated in fibroblasts, platelets, and monocytes, and the level of phosphorylation increases in response to growth factors, activating agents, or tumor promoters (Sardet et al., 1990, 1991b; Livne et al., 1991; Bianchini et al., 1991). It was therefore of interest to analyze the state of phosphorylation of the human antiporter expressed in Sf9 cells and to see whether endogenous protein kinases can respond to agents that easily penetrate intact cells such as tumor promoters. We have used two mechanistically distinct agents: phorbol esters that activate protein kinase C and okadaic acid, which specifically inhibits protein phosphatases PP1 and PP2A (Cohen et al., 1990).

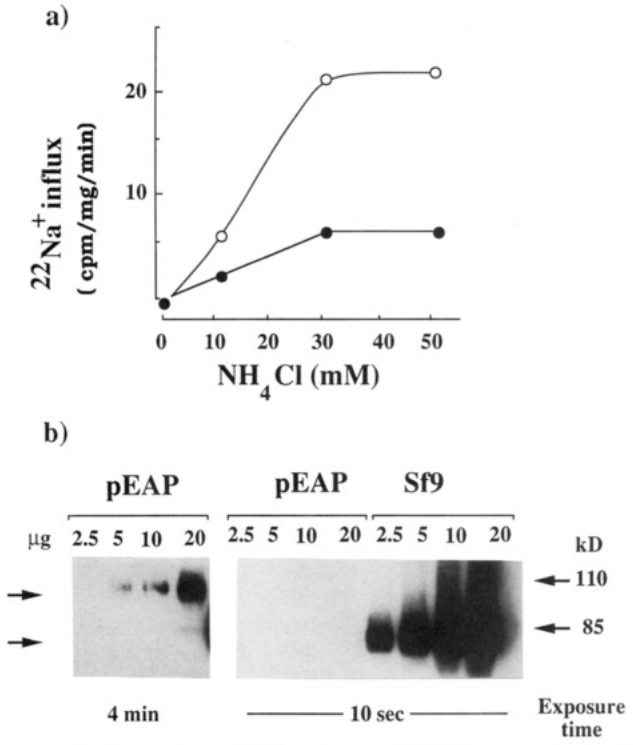

FIGURE 3: Comparison of ^{22}Na uptake and NHE-1 expression levels in Sf9 cells and pEAP fibroblastic cells. (a) ^{22}Na uptake (2 min) was conducted as described under Experimental Procedures except that the cells were H^+-loaded with an increasing concentration of NH_4Cl (0, 10, 30, or 50 mM). The open symbols represent ^{22}Na flux in insect cells 24 h postinfection; the closed symbols represent ^{22}Na flux in the pEAP cell line. (b) Immunological detection of Na^+/H^+ antiporter in insect cells 24 h postinfection and in the pEAP cell line. Cells were washed twice with PBS, and proteins were solubilized in 1% SDS; 20, 10, 5, and 2.5 µg of protein from both cell lines were analyzed by immunoblotting as described in Figure 1 except that detection of the second antibody, labeled with horseradish peroxidase, was conducted by the enhanced chemiluminescence reaction (ECL-Amersham). In the case of the Western blot for pEAP cells, two different times of film exposure are shown (10 s and 4 min).

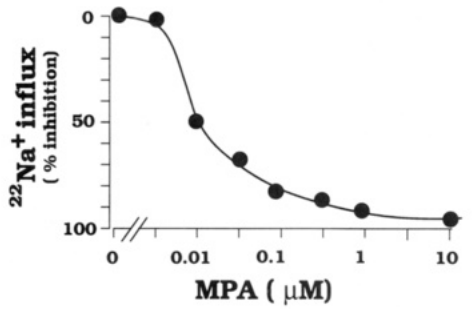

FIGURE 4: Concentration response curve for inhibition of Na^+ uptake by methylpropylamiloride (MPA) in Sf9 cells expressing the human Na^+/H^+ antiporter. Insect cells were first infected for 24 h with BV–NHE-1 recombinant baculovirus, acid-loaded with an NH_4Cl prepulse, and then incubated for 3 min in Na-uptake buffer containing various concentrations of MPA. This representation is the mean from two independent experiments performed in duplicate.

BV–NHE-1-infected cells were incubated overnight in serum-free medium in order to blunt endogenous kinases possibly activated by hormones and growth-promoting agents present in serum. Cells were labeled for 6 h with [^{32}P]orthophosphate in phosphate-free medium and were treated with either PMA, okadaic acid, or solvent as a control. Cells were lysed, the Na^+/H^+ antiporter was immunoprecipitated, and precipitates were analyzed by SDS–PAGE. Figure 5 shows that the antiporter is indeed phosphorylated in insect cells and that, interestingly, both PMA and okadaic acid are able to increase

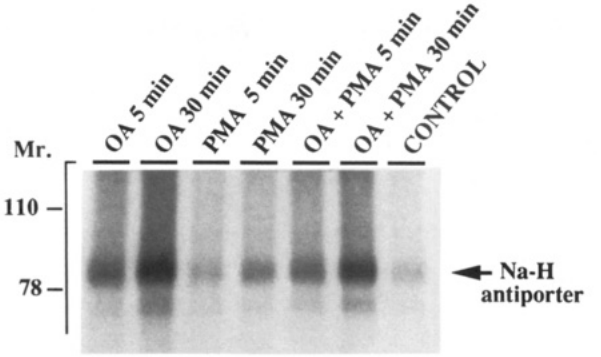

FIGURE 5: Immunoprecipitation of the Na^+/H^+ antiporter from cells labeled with [^{32}P]orthophosphate. Autoradiogram of immunoprecipitates separated by electrophoresis on a SDS–7% polyacrylamide gel under reducing conditions. Cells were infected and labeled as described under Experimental Procedures. After the addition of phorbol ester (PMA, 200 nM) or okadaic acid (OA, 1 µM), reactions were stopped by washing the cells once with ice-cold PBS and freezing the dishes on liquid N_2. Cells were then scraped, solubilized, and immunoprecipitated as already described (Sardet et al., 1990). Lanes 1 and 2: effect of OA for 5 and 30 min, respectively. Lanes 3 and 4: effect of PMA for 5 and 30 min, respectively. Lanes 5 and 6: effect of both OA and PMA for 5 and 30 min, respectively. Lane 7: control cells treated with solvent. Three independent experiments have shown identical patterns of stimulated phosphorylation.

the level of phosphorylation. The effects of PMA were not detectable after 5 min of stimulation. However, a 30-min treatment with PMA increased the phosphorylation of the Na^+/H^+ exchanger about 3 times. Using non-serum-starved cells, we obtained an increased basal level of protein phosphorylation and, as a consequence, a weak stimulation of Na^+/H^+ antiporter phosphorylation by tumor promoters (data not shown).

We recently reported that in Chinese hamster lung fibroblasts, EGF and thrombin stimulate the phosphorylation of the Na^+/H^+ antiporter at serine residues (Sardet et al., 1990). Further, we found that mitogenic stimulation increases ^{32}P incorporation in several peptides with the generation of a common and new phosphopeptide (Sardet et al., 1991a,b). In this context it was of interest to determine the pattern of antiporter phosphorylation in Sf9 cells in response to PMA and to compare it to the corresponding phosphopeptide map of fibroblasts. The two phosphorylated proteins of Sf9 cells (control and PMA-stimulated) were extracted from the gel and digested with trypsin. The phosphopeptides were analyzed by two-dimensional thin-layer electrophoresis/chromatography. Figure 6 reveals identical phosphopeptides with a specific increase in a single phosphopeptide in response to PMA. Interestingly, the phosphopeptide map resembles that obtained from fibroblasts pointing out the same "mitogenically" stimulated phosphopeptide (Sardet et al., 1991b). This result was confirmed by mixing Sf9 and fibroblast-derived tryptic digests. The four major phosphopeptides including the PMA-inducible one comigrate in the two-dimension analysis (data not shown). This somewhat unexpected result indicates a good degree of conservation in the protein kinases network between mammalian fibroblasts and invertebrate cells.

Discussion

The baculovirus expression system has been used successfully to express a wide variety of biologically active proteins, including nuclear, cytoplasmic, membrane-associated, and secreted proteins. Recently, several groups have used this system to express transmembrane receptor proteins such as the β-adrenergic receptor, (George et al., 1989), EGF receptor (Greenfield et al., 1986), and insulin receptor (Paul et al.,

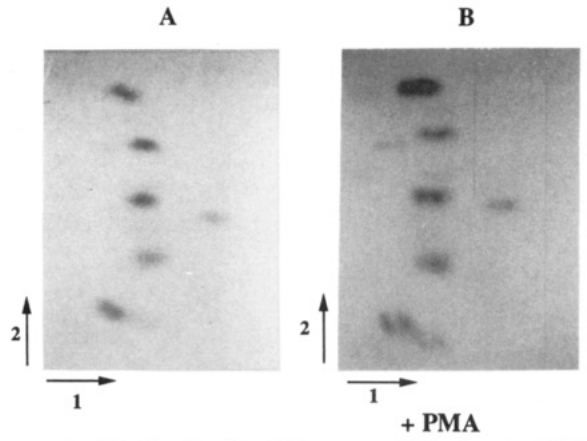

FIGURE 6: Tryptic phosphorylation map of the human Na^+/H^+ antiporter expressed in insect cells. Cell treatment and antiporter immunoprecipitation were carried out as in Figure 5. ^{32}P-labeled proteins were excised from a nondried gel, extracted, and digested with TPCK-treated trypsin as described under Experimental Procedures. The resulting peptides were separated by electrophoresis (arrow 1) followed by thin-layer chromatography (arrow 2) and visualized by autoradiography. (A) Na^+/H^+ exchanger from baculovirus-infected insect cells. (B) Na^+/H^+ exchanger from baculovirus-infected insect cells stimulated for 30 min with 200 nM PMA.

1990) and, more recently, transporters such as multidrug resistance gene product (Germann et al., 1990), sodium–glucose cotransporter (Smith et al., 1991), Na^+/Ca^+ exchanger (K. Philipson, personal communication), and K^+ channel (Klaiber et al., 1990). This paper is the first report showing that the Na^+/H^+ antiporter can be produced in large amounts in insect cells following infection with a recombinant AcNPV that contains the cDNA that encodes the entire human Na^+/H^+-exchanger molecule. The protein produced in insect cells is, in all respects, functionally identical with the human NHE-1 isoform as judged by several criteria. Firstly, the activation of Na–H exchange by intracellular acidification indicates that it fulfills a major role, that is, pH_i regulation. Secondly, the inhibition of the proton-activated Na^+ influx by methylpropylamiloride with an IC_{50} of 10 nM is in good agreement with the value obtained with the human or hamster Na^+/H^+ antiporter. It is therefore suggested that insect cells synthesize and process the Na^+/H^+ antiporter polypeptide in a manner similar to mammalian cells and that the protein is inserted into the plasma membrane in the correct orientation. However, comparing Sf9 cells and the high NHE-1 expressing pEAP cells, we conclude that a small fraction (no more than 10%) of antiport protein reaches the plasma membrane of Sf9 cells in a functional state. Indeed with a 150–200-fold higher level of NHE-1 protein made in Sf9 cells, the V_{Max} for Na^+ transport exceeds only 5-fold that of pEAP cells.

In spite of this high level of expression of NHE-1 protein (above 500-fold that of normal cultured fibroblasts), it is not detectable as a major protein by Coomassie blue staining, indicating that this level of expression remains low compared to soluble protein produced in Sf9 cells (Miller, 1988). Some limitation in the expression could be due to the rapid toxicity, judged by cell death, following 30 h of infection with BV–NHE-1. Toxicity could result from saturation of the processing machinery for integral membrane protein or from ionic gradient imbalance generated by the recombinant protein. Expression of the antiport protein was not significantly increased when the infection was monitored in the presence of the amiloride analogue, suggesting that ionic disturbance is not the cause of limited expression. Two recent examples taken within the same family of proteins, the Na–Ca antiporter and the sodium–glucose cotransporter were functionally expressed in Sf9 cells; one is detectable as a prominent protein, the second is not (E. Wright and K. Philipson, personal communications).

The baculovirus-encoded Na^+/H^+ antiporter has a higher mobility in SDS–PAGE than its mammalian counterpart and corresponds to the mobility of the non-glycosylated in vitro translated polypeptide. Initially mammalian and insect cells both transfer a high mannose oligosaccharide dolichol phosphate precursor to asparagine residues in the nascent polypeptide chain (Hubbard & Ivatt, 1981). Subsequently, mammalian cells trim this high mannose structure to a core oligosaccharide, adding a variety of terminal sugar residues. By comparison, insect cells do not add terminal sugars after trimming the high mannose structure (Miller, 1988). However, it appears that these differences in carbohydrate side chains do not impair the folding or acquisition of tertiary structure of the protein, since transport function, activation by intracellular H^+, and inhibition by amiloride are identical with properties of the fully glycosylated human Na^+/H^+ exchanger.

Both the Na^+/H^+ antiporter produced in insect cells and its authentic counterpart expressed in human platelets, monocytes, and fibroblasts (Sardet et al., 1991) are phosphorylated. This phosphorylation that is increased in response to growth factors and activating agents correlates well with the rise in pH_i that results from Na^+/H^+ antiporter activation (Sardet et al., 1990). It was therefore of interest to see that both tumor promoters, PMA and okadaic acid, increased the phosphorylation state of the recombinant protein produced by insect cells. However, although we failed to detect any pH_i change or amiloride-sensitive Na^+ flux in response to these agents, we surprisingly found a similar pattern of antiporter phosphorylation in fibroblasts and insect cells. Moreover, PMA induced the phosphorylation to the same peptide that is mitogenically phosphorylated in fibroblasts (Sardet et al., 1991). Coinfection of Sf9 cells with BV–NHE-1 and recombinant protein kinase C α baculoviruses did not give a stronger phosphorylation in response to PMA. These results suggest that a PMA-stimulated kinase is fully active in Sf9 insect cells and that the Na^+/H^+ exchanger is a suitable substrate for phosphorylation. In addition, it is tempting to conclude that the pathways involved in the activation of Na^+/H^+ exchange in fibroblasts by phorbol esters are conserved in insect cells. This conclusion is of great value for future studies aimed at identifying the sequence of this "mitogenically" regulated phosphopeptide.

In conclusion, the biosynthesis of recombinant Na^+/H^+ antiporter in insect cells has provided a means for obtaining this molecule in sufficient quantities for biochemical analysis. A large production of recombinant functional Na^+/H^+ antiporter that has retained the properties of the mammalian Na^+/H^+ exchanger and the phosphorylation pattern should facilitate the exploration of different domains of the molecule and the identification of the phosphorylated sites. It is now possible to search for specific kinases that are able to phosphorylate this protein either in vivo by coinfection of Sf9 cells with specific kinases or in vitro with purified protein kinases.

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